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Research Report

Circadian dynamics of vasopressin in mouse selection lines: Translation and release in the SCN

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Abstract

Arg⁸-vasopressin (AVP), a circadian clock-controlled gene product, is released from the hypothalamic suprachiasmatic nuclei (SCN) in mice in a circadian fashion. Previously reported differences in two mouse lines, initially selected for thermoregulatory nest-building behavior (building small nests (S-mice) or big nests (B-mice)) with different circadian organization of behavior and in number of SCN-AVP immunoreactive neurons, were further investigated. We confirmed and expanded the finding that S-mice exhibited constant high levels of SCN-AVP content with no apparent circadian rhythmicity, whereas B-mice had lower numbers of AVP positive cells which varied with time of day. We found that AVP mRNA expression levels at midnight and midday were similar in both lines, as established by in situ hybridization. When AVP transport and release were blocked by colchicine, SCN-AVP immunoreactivity was similar in both lines. This suggests that differences in SCN-AVP content depend on transport or release. Organotypic SCN cultures of B-mice showed more AVP release per neuron than cultures of S-mice. These results reveal that on a mechanistic level the mouse lines differed in transport and/or release of AVP in the SCN, rather than differential regulation of AVP gene transcription or number of AVP immunoreactive neurons.

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Theme: Neural basis of behavior

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Keywords: Nest-building behavior; Clock-controlled gene; House mouse; Vasopressin; Circadian

1. Introduction

Arg⁸-vasopressin (AVP) has been recognized as a clock-controlled output system of the SCN, but its precise role is

still poorly understood [16,22,36,39,43]. Circadian rhythms in SCN-AVP are observed both in vivo and in vitro, at the level of mRNA transcription [29], mRNA quantities [10], peptide content [25,26], and peptide release [30,42,46]. Although an output role of AVP has been hypothesized [22,39], the peptide is not a prerequisite for restoring circadian locomotor rhythms by fetal grafts in SCN-lesioned rats and hamsters [3,18,31] and in SCN-intact old rats [32]. Brattleboro rats lacking AVP expression are behaviorally rhythmic, albeit with a decreased amplitude [38].

Both SCN-AVP content and release correlate significantly with the degree of circadian organization of locomotor behavior in common voles, *Microtus arvalis*. In behaviorally

Abbreviations: AVP, arginine vasopressin; SCN, suprachiasmatic nuclei of the hypothalamus; PVN, paraventricular nucleus of the hypothalamus; SON, supraoptic nucleus of the hypothalamus; S-mice, mice building small nests; B-mice, mice building big nests; ExT, external time; ZT, zeitgeber time; PD, postnatal day; -ir, immunoreactivity; OD, optical density

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non-rhythmic subjects of this rodent species, *in vivo* SCN-AVP content is high and *in vitro* AVP release low with no daily fluctuation in long-term organotypic SCN cultures. In behaviorally rhythmic voles, both *in vivo* SCN-AVP content and *in vitro* AVP release show a circadian rhythm [17,26–28]. Reduced SCN-AVP content correlates with increased loss of circadian rhythmicity in old voles [44]. SCN-AVP content also correlates with more subtle differences in circadian organization of locomotor behavior in house mouse lines bidirectionally selected for thermoregulatory nest-building behavior (mice building small or big nests referred to as S- and B-mice, respectively). Mice with robust circadian organization (S-mice) have a 1.5-fold higher number of SCN-AVP cells than mice with less robust circadian organization (B-mice) [1,7–9]. A similar correlation was found in different rat strains [49], but not in mice selected for high levels of voluntary wheel running activity [20] suggesting a possible role of mouse strain genetic background.

The goal of this study was to examine whether previously reported mouse strain-specific differences in the number of SCN-AVP neurons of S- and B-mice [7] are also present at different time points and whether the cause of such differences could be at the level of AVP translation or AVP release. To this end, we first quantified SCN-AVP content *in vivo* at four time points. In addition, differences in SCN-AVP content between the two mouse lines were investigated at early postnatal times *in vivo* as well as *in vitro* in organotypic SCN cultures. Second, colchicine, a neuronal transport inhibitor, was used to establish the total number of SCN-AVP neurons by detection of cells, which produce AVP even in minimal quantities. Third, we determined AVP mRNA expression levels in the SCN at two points in time. Fourth, to characterize not only translation but also release, we measured AVP release in organotypic SCN cultures of the two mouse lines. Taken together, this study combines *in vivo* and *in vitro* techniques in order to elucidate the circadian AVP output system, from mRNA via production to release of the protein.

2. Material and methods

2.1. Animals

Lines of *Mus musculus*, bidirectionally selected for thermoregulatory nest-building behavior for 56 generations (building small nests (S-mice) or big nests (B-mice)) [4–6,34], and subsequently randomly bred for 37 generations, were used. Only males of these mouse lines were employed for their differences in the number of AVP containing neurons and circadian organization of locomotor behavior [1,7–9]. S-mice (more robust circadian organization of behavior and 1.5-fold more SCN-AVP immunoreactivity (ir)) and B-mice pups were kept with the mother in large cages and as adults were housed individually. Food pellets and tap water were available *ad libitum*. Cages were placed

in a climate-controlled room (20 ± 0.5 °C) in a 12:12 light/dark cycle (lights on at 08:00, 250–350 lux, depending on cage placement). Principles of laboratory animal care (NIH publication No. 86-23, revised 1985) were followed and experiments were approved by the Animal Experimentation Committee of the University of Groningen (DEC No. 2091 and 1093) and University of Alaska Fairbanks Institutional Animal Care and Use Committee (protocol #00-06).

2.2. Experimental protocols

2.2.1. Experiment 1: *in vivo* and *in vitro* SCN-AVP content in adult and immature S-mice and B-mice

Three months old adult male mice ($n = 40$) kept under a 12:12 light/dark cycle were used. Ten mice (5 S- and 5 B-mice) were sacrificed at each of the four time points ExT 0, 6, 12, and 18 (External time (ExT) 0 = middle of darkness [11], corresponding to zeitgeber time (ZT) 18, 0, 6, and 12 (ZT 0 is defined as time of lights on)), respectively, and SCN-AVP content was determined by immunocytochemistry. Mouse SCN-AVP peptide content peaks around ExT 10 and reaches a trough between ExT 18 and ExT 2 in rodents [23–25,41,50]. Therefore, the 4 chosen time points will describe the circadian variation of SCN-AVP peptide content adequately.

SCN-AVP immunoreactivity of pups at PD10 (postnatal day 10 (PD10), day of birth was PD0, $n = 7$ for each line) and juveniles (PD24, $n = 8$ for each line) was compared between S-mice and B-mice. *In vitro* SCN-AVP immunoreactivity after 14 days of organotypic culturing was also compared between the lines. These time points were chosen because PD10 was the age at which pups were sacrificed for organotypic SCN culturing in experiment 4, while PD24 represents a similar age to the time point at which SCN-AVP release was evaluated in organotypic culturing. All animals were sacrificed between ExT 8 and 10.

2.2.2. AVP immunocytochemistry

Animals were deeply anesthetized with an overdose of sodium pentobarbital (100 mg/kg, intraperitoneal.) and killed by decapitation, followed by a quick dissection of the brain. Thereafter, brains were immersion fixed for 24 h in 4% paraformaldehyde in 0.1 M phosphate buffer (PFA, pH 7.4) at 4 °C. Subsequently, brains were stored in 0.01 M phosphate buffered saline (PBS, pH 7.4) with 0.1% NaN_3 . Before sectioning on a cryostat, brains were cryoprotected in 30% phosphate buffered sucrose for 24 h. Coronal brain sections were cut at a thickness of 25 μm , and collected in three sets of evenly spaced sections and stored in PBS with 0.1% NaN_3 at 4 °C until further processing. Brain sections were rinsed in PBS and quenched with 0.1% H_2O_2 for 20 min, followed by three rinses with PBS. Thereafter, brain sections were preincubated with 5% normal sheep serum and subsequently incubated overnight with the primary monoclonal antibody mouse anti-AVP (PS41; 1:500; a gift of Dr. Gainer, Bethesda, M.D.; [2]) at room temperature.

Sections were preincubated again with normal sheep serum (5%), and incubated with biotinylated sheep anti-mouse (1:200; Amersham, England) for 2 h at room temperature. After thoroughly rinsing with PBS, the sections were exposed to HRP-conjugated Streptavidin (1:200; Zymed, CA) for 2 h at room temperature. Triton X-100 (0.5%) was added during all incubation steps. Finally, the sections were processed with diaminobenzidine (DAB)-H₂O₂ (30 mg DAB in 100 ml PBS + 0.01% H₂O₂) under visual guidance.

For the organotypic SCN cultures, all immunocytochemical incubation and rinsing steps were done while the culture remained attached to the coverslip in the culture tube. The cultures were immersion fixed in 4% PFA for 2 h at room temperature. Thereafter, cultures were rinsed in PBS, and immunocytochemical staining for AVP was performed.

2.2.3. Quantitative analyses of AVP immunoreactivity

AVP-ir in the SCN was quantified by counting the number of AVP-ir cells [7,17,44,46] and/or by optical densitometry of AVP-ir with a Quantimet 600 image analysis system [46]. Both quantitative analyses were performed “blind” to mouse line, condition, and circadian time point.

In short, AVP-positive SCN cells were counted (left and right hemisphere were summed) in every second section in pups and juveniles, and every third section in adult mice. AVP staining clearly defines the borders of the SCN. The counts in the sections were assigned to seven levels, equally distributed along the rostro-caudal axis within the SCN. Counts of the two sections containing the middle portion of the SCN (mid SCN; rostro-caudal levels 3 and 4) were summed and taken as a measure of SCN-AVP immunoreactivity. Cell counts of AVP-ir cells in organotypic cultures were performed in the area of the neuronal zone containing the SCN (left and right hemispheres were summed). The Optical density (ODs) of AVP-ir in the SCN was measured in the same sections containing the SCN. OD of AVP-ir of brain sections was also determined in the paraventricular nucleus of the hypothalamus (PVN). Two sections containing the mid-PVN were chosen and counts of left and right hemispheres were summed.

The OD is expressed in arbitrary units corresponding to grey levels using a Quantimet 600 image analysis system [46]. The value of background labeling was measured in an area devoid of AVP cell bodies, fibers, or punctae (a hypothalamic region for the brain sections, and a region in the neuronal zone for the organotypic cultures). The OD of the area of interest was related to the background value by the formula $[\text{Optical Density} - \text{Optical Density}_{\text{background}}] / \text{Optical Density}_{\text{background}}$, thus eliminating the variability in background staining among sections.

2.2.4. Experiment 2: in vivo colchicine injections in S-mice and B-mice

Colchicine was injected into the lateral ventricle to abolish axonal transport of AVP and SCN-AVP content was determined by immunocytochemistry.

2.2.5. Colchicine injections and AVP immunocytochemistry

Accumulation of AVP in the cell body was induced by injection of colchicine, which depolymerizes microtubules preventing AVP transport from the cell body to the terminals [33]. Mice were anesthetized by intraperitoneal injection of ketamine (ketaset 76 mg/kg) and medetomidine (Domitor 1 mg/kg). Using standard sterile surgical procedures, colchicine injections (20 µg total) into the lateral ventricle were performed using the following stereotaxic coordinates: 1.0 mm anterior to bregma, 0.7 mm lateral to the midline, and 2.8 mm below the top of the skull. After completion of the surgical procedure, the anesthetic was reversed by subcutaneous injection of atipamezole hydrochloride (Antiseden, 1 mg/kg). The animals were placed on a heated water blanket throughout the surgery and until fully ambulatory. After recovery from anesthesia, the mice were immediately returned to the original housing conditions. Non-injected control mice were housed individually and disturbance was kept at a minimum before perfusion after 24 or 48 h following the injection. The 24- and 48-h time points did not differ for both lines (S-mice: 1742 ± 101 and 1621 ± 92 ; B-mice: 1746 ± 35 and 1675 ± 55 , respectively) and were pooled for analysis. Anesthesia for perfusion was given between ExT 14 and 15 with minimal disturbance and noise.

For perfusion, mice were deeply anesthetized by intraperitoneal injection of sodium pentobarbital (100 mg/kg) and perfused intracardially with 0.9% saline followed by 4% PFA in 0.1 M PBS buffer, pH 7.3. Mice were perfused in pairs of one S-mouse and one B-mouse. After perfusion, brains were removed and postfixed for 1–2 h and subsequently cryoprotected in 30% phosphate buffered sucrose at 4 °C. Coronal sections of 20 µm were cut on a cryostat microtome. AVP immunocytochemistry was performed as described in experiment 1 with the exception that the secondary antibody was replaced by a biotinylated goat anti-mouse IgG (Vector 1:200) and subsequently exposed to avidin/biotinylated horseradish peroxidase macromolecular complex (Vector, Vectastain ABC kit) and triton X-100 (0.1%) for 30 min at room temperature. DAB processing was done as described in experiment 1. SCN-AVP neuronal counts were performed as described in experiment 1.

2.2.6. Experiment 3: in vivo SCN-AVP mRNA expression in the S-mice and B-mice

Transcription levels of AVP mRNA in the SCN were measured at mid dark (ExT 0) and mid light period (ExT 12), at which time SCN-AVP mRNA expression reaches near peak and near trough expression levels [10,12].

2.2.7. AVP-mRNA hybridization

AVP mRNA hybridization was kindly carried out by Dr. L. Yan and Dr. R. Silver; Columbia University, NY USA. The AVP cDNA fragment-containing vectors (gift of Dr. H. Okamura; Kobe University, Japan) were linearized with restriction enzymes (*Pst*I, Promega) and used as templates for sense or anti-sense cRNA probes. Digoxigenin-labeled

probes were made using Digoxigenin-UTP (Boehringer Mannheim, Mannheim, Germany) with a standard protocol for cRNA synthesis [52]. Sense AVP probe revealed no specific hybridization signals in brain sections (data not shown).

Serial coronal sections (40 μm) were made from the rostral to the caudal end of the SCN using a cryostat (Reichert-Jung, Heidelberg, Germany), alternate sections were processed in situ. To minimize variations in the hybridization procedure, sections from the two mouse lines were processed simultaneously in the same well, by uniquely marking individual brains with different cortical knife cuts. The in situ hybridization histochemistry was performed as described previously [51].

For quantification of optical density, images of serial sections were captured using a CCD video camera (Sony XC77) attached to a light microscope (BH-2; Olympus Optical, Tokyo, Japan). Expression of mRNA was quantified using the NIH Image program (version 1.61). The grey value per pixel was averaged and used to quantify the intensity of the signal in the SCN. For each animal, each bilateral SCN was evaluated in 3 sections taken from the mid-SCN region and the average for each animal was used for statistical evaluation. The SCN was delineated based on the outline of AVP mRNA staining and optic chiasm. A hypothalamic area adjacent to the SCN, four times as big as the SCN, was used to measure background. Relative optical density was calculated as $[\text{Optical Density} - \text{Optical Density}_{\text{background}}] / \text{Optical Density}_{\text{background}}$ [9].

2.2.8. Experiment 4: SCN-AVP release in organotypic cultures of *S*-mice and *B*-mice

Organotypic SCN cultures were made from 10-day-old pups. After 12 days, medium samples were taken every 2 h for AVP release measurements during a 50-h period.

2.2.9. Preparation of organotypic SCN cultures

SCN explants were performed according to the organotypic slice culture technique using the roller-drum method under sterile conditions [15,45]. In short, male pups were decapitated at PD10. The brains were quickly removed and a block of hypothalamic tissue containing the bilateral SCN was cut coronally in 400 μm thick slices using a tissue chopper. Slices containing the SCN were trimmed. The absence of PVN and SON tissue was confirmed by AVP immunocytochemistry (data not shown). The trimmed slices were placed in Gey's balanced salt solution, and cooled for 2 h at 4 °C. Slices were embedded in a plasma clot (10 μl chicken plasma and 10 μl thrombin) on a coverslip and placed in a culture tube with 700 μl of culture medium (25% horse serum, 45% Eagle's basal medium with 62 mM D-glucose and 4.16 mM NaHCO_3 , and 30% Hanks balanced salt solution with 4.16 mM NaHCO_3). The SCN slice was then cultured at 36 °C and rotated at 12 revolutions/h. The medium was replaced once or twice a week. The culture stability after 11 days [15,45] indicates that most cell death

has occurred prior to the sampling period. Drawings as well as photomicrographs were made by phase-contrast microscopy. After 11 days of culturing, culture medium was replaced by sample medium (10% horse serum, 60% Eagle's basal medium, and 30% Hanks' balanced salt solution). Twenty-four hours later, samples were collected from each culture by changing the sample medium (700 μl) every 2 h for 2 days (25 time points). The collected medium was immediately frozen in liquid nitrogen and stored at –20 °C until analysis. Three out of nine cultures from each line did not remain intact, i.e., pieces of tissue had detached from the coverslip, after the sampling period (50 h). At the end of the experiment (14 days after brain slice preparation), SCN cultures were immediately fixed in 4% PFA while remaining attached to the coverslip and subjected to AVP immunocytochemistry. AVP immunocytochemistry was performed while the cultures remained attached to the coverslip (as described earlier). AVP release per neuron was calculated by dividing the total release by the total number of AVP immunoreactive cells.

2.2.10. Radioimmunoassay for AVP

Radioimmunoassay (RIA) for AVP was performed as described before [27,46]. 240 μl of each sample was analyzed using a highly sensitive RIA for AVP according to Watanabe et al. [48], with minor modifications. In short, we used rabbit anti-AVP ("Peter"; The Netherlands Institute for Brain Research, Amsterdam) in a final dilution of 1:16,000. For each tube, 50 μl of SAC-CEL solid phase secondary antibody coated cellulose suspension (IDS, England) was used as precipitation reagent. Standard curves ranged from 0.25 to 64 pg AVP per 50 μl and the assay had a detection limit of 0.25 pg/ml. Cross-reactivity of "Peter" besides AVP was 0.04% with vasotocin, and <0.01% with oxytocin. 240 μl of all samples was analyzed in triplicate (80 μl per tube). The interspecific assay coefficient of variation was 12%. Individual RIA values within a triplicate were discarded when the value deviated more than 7% from triplicate mean. This arbitrary criterion was chosen based on the variation found in all triplicate RIA values.

2.2.11. Analysis of *in vitro* AVP release

An acknowledged mathematical algorithm for detecting peaks in hormonal release is the CLUSTER pulse detection algorithm [47]. CLUSTER analysis detects peaks by performing pooled *t* tests on a bipartite sliding bin (cluster). We modified the algorithm by introducing a minimum inter-bin gap of 2 sample points, in order to take into account the width of nadirs and peaks. This resulted in a 3×3 comparison of nadir and peak samples ($\alpha = 0.05$, $df = 4$), with 2 sample points in between. A peak was defined as the period between a significant increase and the first following significant decrease. Number of peaks and duration of peaks were identified for both lines.

The amplitude of AVP release was measured by taking the difference between maximal and minimal release. Autocor-

relation analysis was performed on individual culture data with post hoc Friedman repeated-measure ANOVA to determine the presence of significant period values.

3. Results

3.1. Experiment 1: *in vivo* and *in vitro* SCN-AVP content in adult and immature S-mice and B-mice

Numbers of AVP-ir positive cells *in vivo* were compared at four time points (ExT 6, 12, 18, and 0; corresponding to respectively zeitgeber time 0, 6, 12, and 18 [11]) using adult mice. No circadian differences were found in the S-mice (Fig. 1). In contrast, B-mice showed circadian variation (ANOVA time effect; $P < 0.001$), with higher numbers of AVP-ir cells at the onset of the light period (ExT 6; Tukey; $P < 0.05$; Fig. 1). The differences observed between the lines in number of AVP-ir cells were an effect seen in the whole SCN and could not be attributed to a possible subdivision (ventro-lateral or dorso-medial) of the SCN. Region specificity and quality of the staining technique were confirmed by inspection of the PVN of the hypothalamus, which revealed abundant AVP immunoreactivity. The PVN did not reveal fluctuations in AVP content in the light period (mean ODs at ExT 6, 12, and 18: for S-mice: 2.09, 2.67, 2.42, and for B-mice: 2.22, 2.40, and 1.91, respectively) showing that fluctuations in the SCN-AVP-ir were region-specific.

The SCN-AVP system of mice at PD10 and PD24 was compared between the S-mice and the B-mice. In both

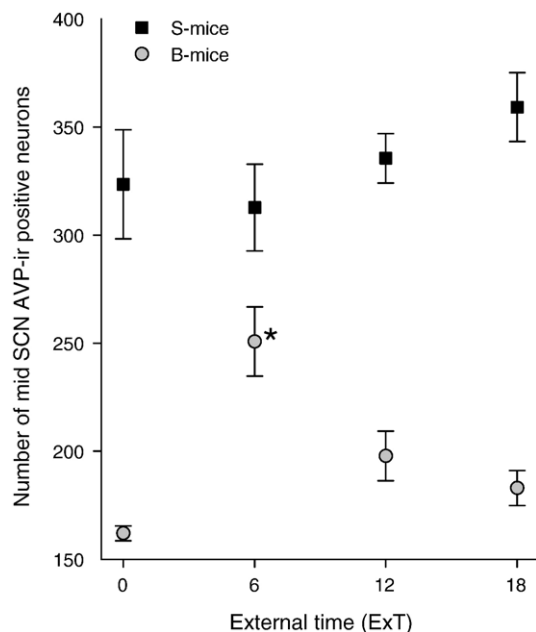


Fig. 1. Quantification of the number of AVP-positive cells in the SCN of S-mice (black squares) and B-mice (grey circles) at ExT 0, 6, 12, and 18 ($n = 5$ per ExT and mouse line). Asterisk indicates a significant difference at ExT 6 for B-mice (Tukey; $P < 0.05$). Two-way ANOVA revealed an overall difference between the B-mice and S-mice where the S-mice showed significant ($P < 0.05$) higher numbers of AVP-ir positive neurons.

mouse lines, at PD10, AVP-ir somata were distributed similarly as seen at PD24 and in adulthood. A large cluster of AVP-positive cells was present along the dorsomedial SCN (“SCN-shell”, [35]), and a smaller cluster at the ventrolateral SCN (“SCN-core”, data not shown). At PD10, however, AVP-ir in cell bodies and fibers was low in both lines, and no densely stained cells were yet present in contrast to PD24. Quantitative analyses revealed no differences in AVP expression between the lines at PD10, neither in OD of AVP-ir neurons (Fig. 2A) nor in number of AVP-positive neurons (mean values \pm SEM for OD at PD10 for S-mice = 368 ± 55 , for B-mice = 300 ± 27 , MWU-test; $P > 0.05$). Significant differences between the lines were present at PD24, where S-mice had 47% higher AVP OD values (t test; $P < 0.05$; Fig. 2) and a 49% larger number of SCN-AVP neurons (S-mice 661 ± 17 ; B-mice 445 ± 7 ; MWU-test; $P < 0.001$). AVP content in the mid SCN was significantly higher at PD24 compared to PD10 within both lines (MWU-test; $P < 0.01$ for both lines).

Comparison of AVP-ir in organotypic cultures between the two mouse lines was performed 14 days after the start of the culture, which is comparable to PD24, on cultures that remained intact after 50-h of sampling for AVP release (6 out of 9 for each line). Two types of AVP-ir neurons were discerned; densely and lightly stained cells (Table 1). No difference in the number of lightly stained cells was found between the lines (MWU-test; $P = 0.2298$). S-mice cultures contained significantly higher numbers of densely stained cells in the neuronal zone than B-mice cultures (MWU-test; $P < 0.01$). As a consequence, the total number of AVP cells also differed between the lines (MWU-test; $P < 0.01$). The OD values of AVP-ir were significantly higher (approximately 2-fold) in S-mouse cultures compared to B-mouse cultures (MWU-test; $P < 0.05$; Table 1).

3.2. Experiment 2: *in vivo* colchicine injections in S-mice and B-mice

AVP-ir cell counts of each of the 7 sections through the SCN showed significant differences (ANOVA; $P < 0.001$), where in non-injected control mice the S-mice had consistently higher numbers of AVP-ir neurons than the B-mice (Tukey t test; $P < 0.001$), while the counts from the colchicine-injected mice were similar for both lines (Fig. 3). After colchicine injection, a significantly higher accumulation of AVP in the SCN of B-mice occurred (Tukey t test; $P < 0.001$) raising it to the same level as S-mice, while in S-mice, no significant rise in cellular AVP was seen.

3.3. Experiment 3: *in vivo* SCN-AVP mRNA expression in the S-mice and B-mice

SCN-AVP mRNA levels were evaluated by *in situ* hybridization at midsubjective day (ExT 12) and midsubjective night (ExT 24) (Fig. 4). *In situ* hybridization revealed that AVP mRNA levels in the SCN did not differ between the

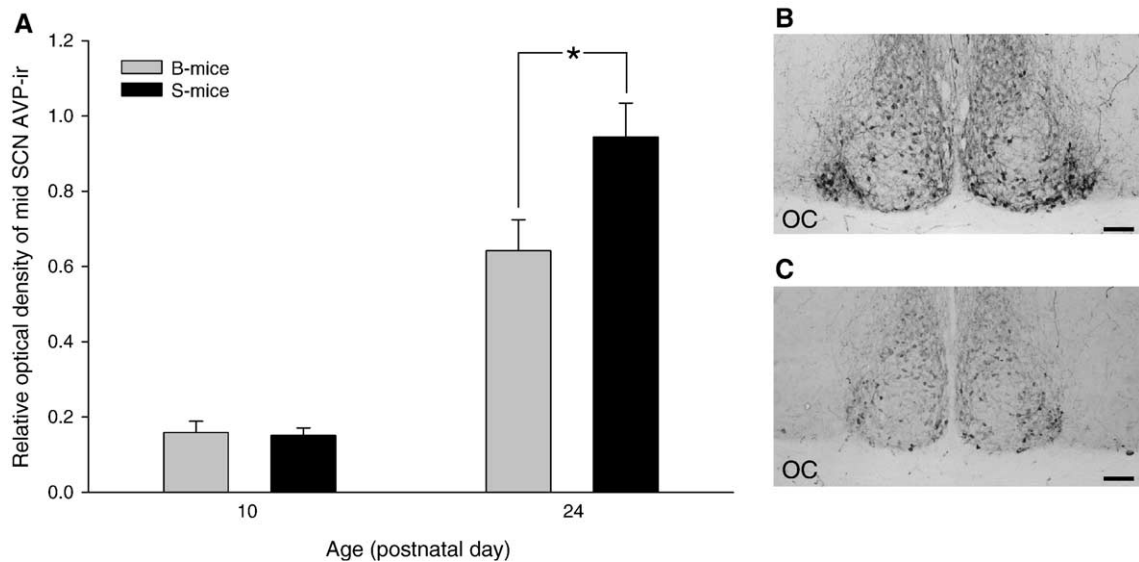


Fig. 2. Quantification (A) of AVP-ir in the mid-SCN region of pups at PD10 ($n = 7$ per mouse line) and juveniles at PD24 ($n = 8$ per mouse line) of B-mice (grey bars) and S-mice (black bars). Panels B and C show photomicrographs depicting AVP-ir in the SCN in juvenile (PD24) S-mice (B) and B-mice (C). Asterisk depicts a significant difference between the two lines at PD24 ($P < 0.05$). OC = optic chiasm; scale bar = 100 μm .

two mouse lines. However, levels of mRNA were significantly lower at ExT 24 than at ExT 12 (two-way ANOVA; $P < 0.0001$). These data are consistent with published work that shows a peak of SCN-AVP mRNA expression around ExT 14 and a trough around ExT 2 [10,12].

3.4. Experiment 4: SCN-AVP release in organotypic cultures of S-mice and B-mice

SCN tissue from S-mice and B-mice was explanted at PD10. 12 days after explanting, AVP release from SCN cultures was measured every 2 h over a period of 50 h.

Representative AVP release profiles (expressed as percentage of the mean) of both S-mouse and B-mouse cultures are presented in Fig. 5. CLUSTER analysis revealed at least one, and never more than 3 peaks in AVP release per culture. B-mouse cultures showed an average of 1.5 peaks per culture during the 50 h sample period, with an average duration of 3.9 (± 2.3) h. S-mouse cultures showed 2 peaks on average, with a duration of 4.2 (± 1.7) h. Neither number of peaks (MWU-test; $P > 0.05$) nor duration of peaks (MWU-test; $P > 0.05$) were significantly different between the lines (Table 1). The average amplitude of the largest AVP peak per culture was approximately 2-fold higher in B-mouse cultures compared to S-mouse cultures, but did not

Table 1
Changes of the SCN-AVP system in organotypic SCN cultures

	S-mice	B-mice
<i>AVP release</i>		
Averaged number of peaks in 50 h	2.0 \pm 0.9	1.5 \pm 0.5
Averaged duration of AVP peak (h)	4.2 (± 1.7)	3.9 (± 2.3)
Averaged amplitude of largest AVP peak (pg)	65.70 \pm 15.72	125.70 \pm 53.91
# cultures with period according to auto correlation	2	5
Averaged period (h)	23.0 \pm 1.41	26.4 \pm 1.30
<i>AVP-ir</i>		
Number of SCN-AVP-positive cells:		
Densely stained cells	144.2 \pm 20.7**	38.2 \pm 12.4
Weakly stained cells	100.5 \pm 13.6	79.0 \pm 11.1
Total stained cells	244.7 \pm 16.7**	117.2 \pm 15.1
AVP content in neuronal zone containing the SCN (OD)	0.48 \pm 0.10*	0.20 \pm 0.04
Surface area of neuronal zone (*10 ⁵ μm^2)	1.16 \pm 0.19	1.16 \pm 0.11

* $P < 0.05$.

** $P < 0.01$.

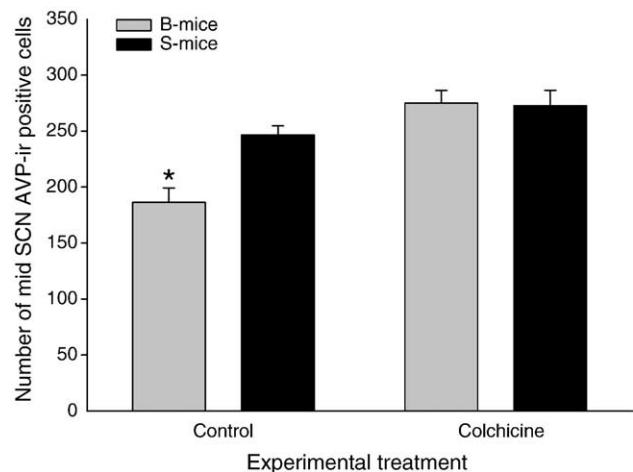


Fig. 3. Quantification of AVP-positive cells in the SCN of control and colchicine injected B-mice (grey bars) and S-mice (black bars) ($n = 9-12$ per mouse line, per condition). Asterisk depicts a significant difference between control B-mice and other groups (ANOVA; $P < 0.001$; Tukey; $P < 0.001$).

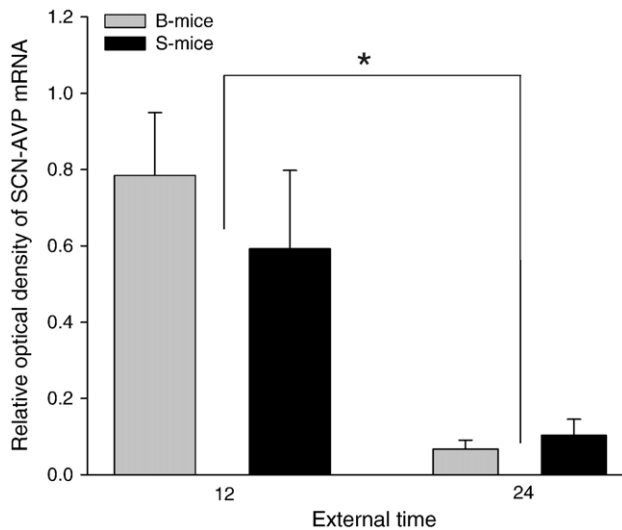


Fig. 4. Relative optical density measures of AVP mRNA in the SCN of B-mice (grey bars) and S-mice (black bars) at ExT 12 and ExT 24. Asterisk depicts a significant ($P < 0.0001$) difference between ExT 12 and ExT 24 in both lines.

differ significantly (125.82 and 65.70 pg AVP in B-mouse and S-mouse cultures, respectively; MWU-test; $P > 0.1$; Table 1). Autocorrelation analysis revealed that, in those cases where a circadian period could be calculated, the average period in S-mouse cultures was shorter than that in B-mouse cultures (Table 1).

Total AVP release over 50 h was determined by summation of all 2-h samples. B-mouse cultures showed a 34% higher release than S-mouse cultures, but this was not significantly different (310.2 ± 43.43 versus 230.8 ± 45.1 pg in 50 h for B-mouse and S-mouse cultures, respectively; MWU-test; $P > 0.05$).

SCN-AVP immunoreactivity performed on the cultures, after the 50 h sampling for release, indicated that the S-mice

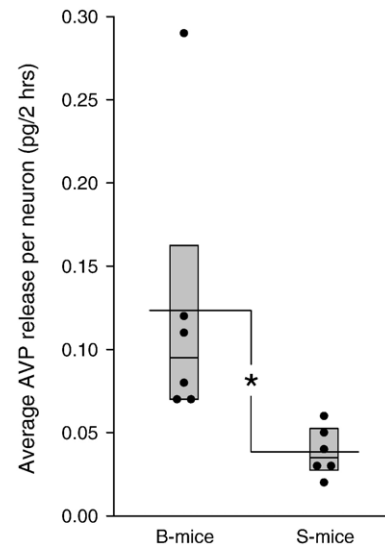


Fig. 6. SCN-AVP release per neuron (expressed in pg/2 h) for individual B-mice and S-mice cultures (black circles) and box plot with median, 25 and 75% interval. Averaged values for B-mice and S-mice cultures (black horizontal lines) reveal a significant higher amount of AVP release per AVP neuron in B-mice, indicated by the asterisk ($*P < 0.01$; MWU).

displayed a significantly higher number of total AVP-ir cells (experiment 1). To take into account this mouse line difference and the variation among cultures, we expressed AVP release per number of AVP-ir neurons (per 2 h) (Fig. 6). B-mouse cultures showed significantly higher (>3-fold) AVP release per neuron than S-mouse cultures (MWU-test; $P < 0.01$; Fig 6).

4. Discussion

Previously, we found a 1.5-fold larger number of SCN-AVP neurons in adult S-mice compared to B-mice [7].

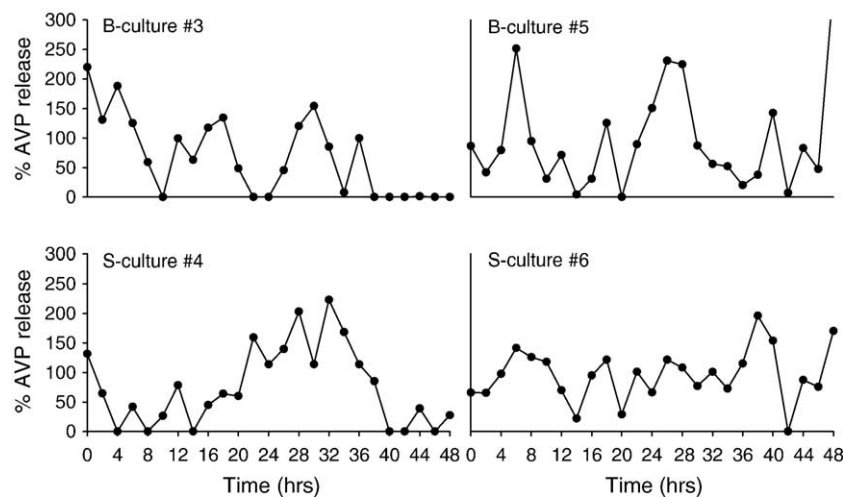


Fig. 5. Examples of AVP release (expressed as the percentage of the mean of AVP release over 50 h), from organotypic SCN cultures made from B-mice and S-mice. Different culture numbers indicate cultures from different mice. Also see Table 1.

These results have been confirmed and expanded in this study including different external times. Differences in the number of SCN AVP-ir neurons between the two mouse lines are not due to different numbers of SCN-AVP producing neurons, because the mouse lines did not differ in the number of SCN-AVP-ir neurons after colchicine treatment. Furthermore, AVP mRNA expression levels were similar between the mouse lines supporting the idea that the difference in the number of SCN AVP-ir neurons is not due to different transcription levels. Organotypic SCN cultures of B-mice showed more AVP release per neuron than cultures of S-mice. Taken together, our results suggest that on a mechanistic level S-mice and B-mice differ in transport and/or release of AVP in the SCN, rather than in regulation of AVP gene transcription or the number of AVP producing cells.

Low amino transmitter content due to a higher level of release has been suggested for a variety of SCN neuropeptides [40,17]. As a possible consequence of release, AVP levels dropped below immunocytochemical detection levels, and therefore the counts of AVP-ir neurons decreased. This interpretation is further supported by the differences in AVP release per AVP neuron in SCN explant cultures of the two lines. However, when SCN-AVP cells drop below immunocytochemical detection levels, caused by high release, it could be that in both S- and B-cultures the same overall numbers of SCN-AVP cells are present, as seen after colchicine injections *in vivo*. In the case of identical numbers of AVP cells in the cultures of either lines, the release per AVP immunoreactive neuron is an overestimation for the cultures made of B-mice, as a result of lower AVP peptide levels. This differential SCN-AVP release or transport also, at least in part, seems to be reflected in the higher amplitude of release in cultures of B-mice (Table 1).

The difference in SCN-AVP content between the S-mice and the B-mice was present as early as PD24, but not at PD10. Although the SCN-AVP system was not fully expressed at PD10, AVP expression in the mouse SCN has been reported as early as embryonic day 16 [21]. SCN-AVP expression increased from PD10 to PD24 in both mouse lines similarly to what has been observed in rat and hamster [13,14]. In contrast, mice derived from BALB/cJ \times C57BL/6JN revealed no further maturation of SCN-AVP expression after PD6, neither in cell number nor in cell size, although a trend in AVP OD increase was present from PD6 to PD30 [19].

Our AVP mRNA results do not support differences in transcriptional control of SCN-AVP between the lines. Of course, we cannot exclude that differences at other time points than the two key time points we chose could be present. The higher level of SCN-AVP release per AVP neuron in the B-mice supports this. Differences in SCN-AVP release could originate at the level of axonal transport and/or release of the AVP peptide at the axon terminal. Indeed, large scale gene expression profiling in mice revealed circadian variation in a number of gene

transcripts coding for proteins involved in presynaptic vesicle availability [37]. One possible explanation is that neurons from the S-mice do not receive or do not respond to stimuli for circadian release. Quantities of AVP accumulation after colchicine injections do not suggest that degradation of AVP is a rate-limiting factor in this model.

High levels of SCN-AVP immunostaining have been observed in non-rhythmic voles compared to rhythmic ones [17,26], and in wild-type hamsters compared to tau-mutant hamsters [46], whereas AVP release was significantly higher in rhythmic voles [27] and tau-mutant hamsters [46]. When high levels of SCN-AVP immunoreactivity indicate low levels of AVP release [17,40], high AVP immunoreactivity reflects a less dynamic SCN-AVP system. Our results support this statement, because we found high levels of AVP immunoreactivity and low levels of AVP release in the SCN of S-mice compared to B-mice.

AVP release in SCN cultures of S- and B-mice do not show differences in number of peaks during the 50 h sampling period. The intrinsic periods of S- and B-mice differ only by 13 min under free-running conditions [8] and this difference has been inconsistent (unpublished data). The amplitude of peak SCN-AVP release is less in S-mice, indicating a less robust circadian rhythm of release. Release per neuron is also less in S-mice compared to B-mice.

Although our results thus indicate that AVP release in the S-mice is less circadian and less abundant, circadian organization of behavior is more pronounced [8,9]. We initially expected that the S-mice would have a more robust circadian system with higher AVP release amplitude and precision in periodicity compared to B-mice, based on previous analyses of circadian rhythmicity in locomotor activity [8,9]. Although the circadian rhythmicity seems to be more pronounced in S-mice, amplitudes of phase responses to a light pulse are less than for B-mice [8]. Possibly, this positive correlation between phase responses and SCN-AVP release is indicative of a different level of control, where higher levels of AVP output increase the susceptibility of the circadian system to shifts. Also in mice selected for high levels of voluntary wheel running, no correlation between SCN-AVP content and circadian organization of wheel-running activity was seen [20], suggesting that in these mice SCN-AVP may not be directly involved in circadian locomotor behavior. The Brattleboro rat, effectively lacking AVP due to a point mutation in the AVP gene, does show circadian organization of behavior, but with a lower amplitude [38]. However, in the common vole SCN-AVP release is positively correlated with robustness of circadian organization of locomotor behavior [17,26–28]. Species-specific correlations are also reflected in protein kinase C signal transduction, which show differential expression, both in levels and circadian variation [[9] and references therein]. In conclusion, although differences in SCN-AVP are strongly correlated with differences in behavior, these correlations are in a different direction

depending on the species, and thus do not reflect a general characteristic of SCN-AVP content.

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